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Echinomycin in the prevention of heterotopic ossification – An experimental antibiotic agent shows promising results in a murine model

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ABSTRACT

Background: Heterotopic ossification (HO) frequently causes complications following orthopaedic and trauma surgery and may drastically reduce the postoperative outcome due to pain and joint contracture. Current therapeutic options include NSAID's and local radiation. However, both options of prevention show disadvantages such as delayed fracture healing and impaired ossification as well as other side effects.[9] Our goal was to investigate a novel approach in the prevention of heterotopic ossification by pharmacologically interfering with the molecular signalling pathways involved in this process.

Hypoxia leads to numerous effects on a cellular level, one of which is the activation of the transcriptional complex hypoxia-inducible factor (HIF).[19] Among several other actions, the HIF1-α signalling pathway in turn regulates angiogenesis through induction of the expression of vascular endothelial growth factor (VEGF).[21]

We hypothesised that by pharmacologically interfering with the HIF-1α signalling pathway, the amount of HO formation may be reduced.

Echinomycin is a known inhibitor of HIF-1-alpha and was used in our study with the aim to prevent HO from forming.

Methods: We examined the effect of Echinomycin on HO formation in a murine model where an Achilles tenotomy was performed. This has previously been shown to reliably produce islets of heterotopic ossification within the soft tissue of mouse hind limbs at 10 weeks after surgery. The control group underwent Achilles tenotomy only, whereas the Echinomycin group additionally received Echinomycin subcutaneously. After trial completion, the limbs were harvested and Micro-CT was performed. Heterotopic bone volume was then identified in 3d images and quantified.

Results: We found a highly significant reduction in the bone volume following subcutaneous administration of Echinomycin compared to the control group.

Conclusion: Although a substantial reduction could be achieved, it was not possible to completely prevent heterotopic ossification from forming. Further studies have yet to be conducted to optimise the results by altering the dosage and duration of administration as well as investigate the mechanism by which Echinomycin led to the reduction of HO formation.

Introduction

Heterotopic ossification (HO) is an important problem throughout orthopaedic surgery.

It is defined as the abnormal formation of lamellar bone within soft tissue and may occur following local trauma or operation.

The origin of HO is believed to lie in dormant osteoprogenitor stem cells within the affected soft tissues. If stimulated, these cells go on to differentiate into osteoblasts and begin the process of osteoid formation, which eventually leads to the formation of mature heterotopic bone.[14] Surgical removal of ectopic bone is the only effective treatment although reoccurrence after excision is frequent. This underlines the importance of an effective prophylactic regimen, of which several have been proposed. Among these, NSAIDs and radiation therapy are most popular. Both may still be of value after surgical excision of HO. However, in primary prevention of HO, their use has been questioned not only because of potentially serious side effects but also because they may be deleterious in a trauma setting or following joint implant procedures as they have been proven to inhibit fracture healing

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and implant ingrowth.[5,6,9] Additionally, current options to prevent HO merely decrease the incidence, but cannot completely prevent their occurrence.

The exact signalling pathways in the pathogenesis of heterotopic bone formation are not yet fully understood due to their complexity. However, numerous contributing factors have emerged so far.

Osteogenesis and angiogenesis are believed to share some common mediators. The replacement of avascular osteoid tissue by highly vascularised bone represents the final event in endochondral ossification of bone growth.[2] Histological findings indicate that osteoblasts and osteoprogenitor cells always develop concomitantly with endothelial cells after being brought into the cartilage templates at sites where new bone is formed. Angiogenic stimulators (e.g. induced by hypoxic stress) might therefore not only influence blood vessel development but also the formation of HO.[22] Recent studies have shown that a hypoxic microenvironment indeed seems to play a key role in HO development.[15] An important signalling pathway activated by hypoxia is the transcriptional complex hypoxia inducible factor (HIF).[19] A further step in the development of heterotopic ossification is the formation of blood vessels, which, amongst others, bring stem cells into the tissue where HO is about to form. An important signalling molecule involved in this process is vascular endothelial growth factor (VEGF). It has been found to be up-regulated – among others – by hypoxia-induced factor (HIF 1 alpha).[21]

Liu et al. showed that HIF-1 can regulate the bone formation ability of osteoblasts in postmenopausal osteoporosis. Low oxygen tension may critically influence chondrocyte differentiation by accelerating the growth of mesenchymal stem cells and promoting their commitment to the chondrocyte lineage, in part, by up-regulating a programme of chondrocyte-specific gene expression under the control of hypoxia-inducible factor 1.[11]

Echinomycin is an antibiotic agent derived from the quinoxaline family, which inhibits HIF1, a DNA-binding activity.[10] It has not only been investigated for its ability of HIF-1-alpha inhibition, but was originally discovered as an antibiotic agent showing similar activity compared to Vancomycin in staphylococcal infections. With this in mind, if proven to effectively prevent HO, Echinomycin may be of additional value in a clinical setting where a postoperative antibiotic prophylaxis is necessary.[17]

The major outcome of interest of this investigation was to study the amount of HO formed under the effect of a substance that inhibits HIF-1alpha to indirectly gain insight on the signalling involved in ectopic bone formation in a well-established murine model.

Materials and methods

Animal model

The study was approved by the relevant Swiss authorities. The model used[13] is a well-established murine model in which HO formation has been shown to reliably produce HO within the soft tissue of the murine hind limb following Achilles tendon tenotomy at ten weeks after surgery.

Although the model described does not necessitate a specific strain or breed of mice, only males have been used (since in humans, HO is more predominant in males and since males have also been used in other similar models). CD1 mice were selected because they are bred locally, are somewhat larger animals, and are not genetically modified. Identification was carried out by markings made on the tail.

Anaesthesia was carried out using Isoflurane 5–2% in oxygen (flow rate 400 ml/min) via nose cone, combined with Temgesic subcutaneously.

The anesthetised mice underwent bilateral midpoint Achilles tendon tenotomy through a posterior approach, and the skin was closed using non-absorbable sutures. Following this procedure, the animals were randomly assigned to one of two groups: A control group ($n = 10$) and a treatment group ($n = 10$). Perioperative analgesia was mainly paracetamol (Dafalgan syrup 3%, 200 mg/kg), for 1–3 days [from Laboratory Animal anaesthesia, von Paul Flecknell, second edition, 1996].

To reduce stress and possibly pain postoperatively, the animals were checked several times a day. If they presented signs of postoperative distress (apathy, shivering, no chow and water intake) the treatment with paracetamol was extended. A score-sheet was developed for detection of discomfort.

Treatment

The control group underwent Achilles tenotomy only. The treatment group additionally received 10 mcg/kg (0.3 mg/kg body weight) Echinomycin diluted in DMSO by means of a subcutaneous interscapular injection once a week for 4 weeks, followed by 6 weeks of rest and cage activity only.

Assessment

At ten weeks after surgery, the mice were euthanised and the limbs harvested. Radiographic work-up for the presence of HO was carried out using Micro CT of both hind legs with a nominal resolution of 30 μm (b-cube, Swiss Federal Institute of Technology, Zurich, Switzerland). The volume of heterotopic mineralised tissue was then calculated with use of the quantitative bone analysis software provided with the micro-CT system. First, 2-dimensional overview images were generated. Those images showed a series of slices through the specimen. Skeletal – as well as ectopic bone was separated from background by a fixed thresholding procedure. The heterotopic bone compartments were then manually identified and analysed as a separate compartment. HO was defined as any bone within soft tissue with a density at least equivalent to that of spongy skeletal bone. Following this, a three-dimensional image of each limb was created and the volume of heterotopic bone could be identified and quantitatively assessed. Statistical analysis was conducted in collaboration with the Division of Biostatistics at the Institute for Social and Preventive Medicine of the University of Zurich, Switzerland. The analysis was performed using SPSS software (IBM, Chicago, IL). Bone volume was analysed using descriptive statistics (ANOVA) and differences between the groups were identified using the Wilcoxon rank sum test. Data were given as heterotopic bone volume in mm^3, the level of significance set at $p < 0.05$. The histological stainings where semiquantitively assessed.

Histology

The harvested limbs additionally underwent immunohistochemical staining using a HIF-1-alpha antibody. Mice hind limb specimen were trimmed above the knee and below the ankle. To demineralise the bone before histological preparation, trimmed specimens were bathed in Tris-EDTA pH 7.0 solution for 21 days and embedded in paraffin before being cut to 7 μm slices. The cut samples were de-paraffinised in Xylol and slewed in an alcoholic series. Then they were counter-stained with Hemalaun. The antigen was enzymatically exposed through Proteinase K, leaving the samples at room temperature for 10 min (DAKO Real Proteinase K (40×) S2019, Diluent S2032). The endogenic Peroxidase was inhibited by hydrogen peroxide 3% for 10 min at room temperature. Then the samples were treated against unspecificities with the Dako Protein block (Dako X0909).